

Inhibition of *Legionella pneumophila* Multiplication within Human Macrophages by Antimicrobial Agents

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The activity of serial concentrations of different antimicrobial agents on the multiplication of *Legionella pneumophila* within human monocyte-derived macrophages was studied. The results led to the definition of a minimal extracellular concentration inhibiting intracellular multiplication (MIEC). According to the MIECs, the antimicrobial agents tested were classified in three groups: (i) very active (MIEC ≤ 0.06 $\mu\text{g/ml}$), such as erythromycin, rifampin, and pefloxacin; (ii) active (1 $\mu\text{g/ml} \geq \text{MIEC} \geq 0.1$ $\mu\text{g/ml}$), such as sulfamethoxazole-trimethoprim or doxycycline; and (iii) ineffective, such as cefoxitin, which was not active within macrophages at as high as 64 $\mu\text{g/ml}$ despite a low MIC (0.2 $\mu\text{g/ml}$) on bacterial charcoal-yeast extract agar. The activity of netilmicin was difficult to assess because of its effect on extracellular legionellae. Combinations of erythromycin with rifampin and pefloxacin with erythromycin, rifampin, doxycycline, or netilmicin showed an additive effect and no antagonism. These results obtained in a cellular model are in agreement with the efficacy of antimicrobial agents in experimental infections and in Legionnaires disease. They sustain clinical interest in the new quinolones, such as pefloxacin, and in combinations of antimicrobial agents for the treatment of Legionnaires disease.

Legionella pneumophila, the agent of Legionnaires disease, is a facultative intracellular bacterium which multiplies within phagocytic cells (13). Erythromycin alone or in combination with rifampin is the currently accepted treatment of Legionnaires disease, though failures or delayed activity are observed, especially in immunocompromised patients (2, 18, 21). Thus, the activity of other antimicrobial agents has to be tested. However, the assessment of in vitro antibiotic activity by conventional means is misleading because of (i) partial inhibition of some antibiotics by a component(s) of the media used for *Legionella* growth (3, 6, 16) and (ii) the discrepancy between in vivo inefficacy and in vitro low MICs of some antibiotics, especially β -lactams, caused by a low intracellular penetration at the site where *Legionella* sp. multiplies.

To overcome these obstacles, we used a cell model to assess the activity of antibiotics against *Legionella* sp. multiplying within macrophages. The effect of decreasing concentrations of the anti-infectious agents erythromycin, rifampin, doxycycline, netilmicin, sulfamethoxazole-trimethoprim, pefloxacin, and cefoxitin led to the definition, for each of them, of a minimal extracellular concentration inhibiting the intracellular multiplication (MIEC) of *L. pneumophila*. Moreover, our results sustain the use of antibiotic combinations and the potential interest of new quinolones such as pefloxacin in the treatment of Legionnaires disease.

MATERIALS AND METHODS

Bacteria. *L. pneumophila* serogroup 1, strain Paris/CB 81-13, was used in all experiments. This strain was isolated from the lung of a patient who died from Legionnaires disease, and it was plated on bacterial charcoal-yeast extract (BCYE) agar only twice before use. Susceptibility of this strain to antimicrobial agents was compared with the sus-

ceptibility of eight other *L. pneumophila* serogroup 1 strains isolated from patients and of the *L. pneumophila* serogroup 1 reference strain ATCC 33152. In vitro antibiotic susceptibility tests were performed by agar dilution with a Steers replicator (3). The medium was BCYE agar with appropriate concentrations of the agents. For the inoculum, a 48-h culture was taken from BCYE agar, suspended in distilled water, and diluted to 1×10^8 to 6×10^8 CFU/ml. Each inoculum spot contained 10^5 to 10^6 CFU.

Sera. Pooled normal human serum was collected from five healthy donors. Immune serum was obtained from a nonimmunocompromised patient with culture-proven Legionnaires disease; the antibody titer to *L. pneumophila* serogroup 1 was 1:512 as determined by an immunofluorescence test.

Monocyte-derived macrophages. Human monocyte-derived macrophages were obtained as described previously (25) from the peripheral blood of a healthy donor who was seronegative ($<1:16$) for *L. pneumophila* serogroup 1. Briefly, 15 ml of venous blood mixed with 5 ml of 3% gelatin (Plasmagel; Roger Bellon, Neuilly, France) and 50 μg of heparin per ml was sedimented at 37°C for 45 min. The leukocyte-rich plasma (2 ml) was poured into Leighton tubes. Between 5 and 12 tubes were used in each experiment. After sedimentation for 3 h at 37°C , the plasma containing nonadherent cells was removed, and adherent cells were incubated with antimicrobial agent-free TC 199 medium (Hanks base) containing 10% normal serum and 50 μg of heparin per ml. This nutrient medium without heparin was changed on days 1 and 3, and tubes were incubated with 5% CO_2 . On day 7, a homogeneous monolayer of well-spread macrophages was obtained.

Antimicrobial agents. The following injectable forms of antimicrobial agents were used: erythromycin lactobionate (Abbott Laboratories, North Chicago, Ill.), rifampin formaldehyde sulfoxylate (Lepetit, Milan, Italy), doxycycline (Pf-

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TABLE 1. MICs of antimicrobial agents against *L. pneumophila*

Antimicrobial agent	MICs on BCYE agar ($\mu\text{g/ml}$)		
	Strain		Eight strains from patients
	Paris CB 81.13	ATCC 33152	
Erythromycin	0.5	0.5	0.06–0.5
Rifampin	0.05	0.03	0.001–0.12
Doxycycline	1	1	0.5–2
Pefloxacin	0.5	1	0.12–1
Netilmicin	0.5	0.5	0.1–1
Cefoxitin	0.2	0.1	0.1–2

izer Inc., New York, N.Y.), sulfamethoxazole-trimethoprim (Roche Diagnostics, Div. Hoffman-La Roche, Inc., Nutley, N.J.), pefloxacin mesylate dihydrate (Roger Bellon), netilmicin (Unilabo, Levallois-Perret, France), cefoxitin sodium (Merck Sharp & Dohme, West Point, Pa.).

Infection of macrophage monolayers. Inoculation of macrophages was performed on day 7 of culture. *L. pneumophila* in TC 199 with 10% immune serum was added to macrophages in a ratio of 100 ± 10 bacteria per cell. At 60 min, extracellular and intracellular bacteria were enumerated in one of the Leighton tubes. Extracellular bacteria were removed by several washings with a determined volume of phosphate-buffered saline (with Ca^{2+} ions; pH 7.2); thereafter, intracellular bacteria were counted after disruption of macrophages by introduction of 2 ml of distilled water over 30 min and by mechanical shaking. These procedures did not alter the viability of bacteria. In other tubes, the supernatant containing noningested bacteria was removed, and the adhering bacteria were washed out with phosphate-buffered saline. TC 199 medium (2 ml) with 10% normal human serum and various concentrations of antimicrobial agents, alone or in combination, was then added; the total volume was 2 ml per tube. In each experiment, a control tube without agent was included. In an earlier study, we showed that, at 60 min, bacteria were readily ingested and that after washings with phosphate-buffered saline, no cell-bound legionellae were demonstrable morphologically by direct fluorescence assay (24).

Extra- and intracellular bacteria were enumerated after incubation at 37°C for 24 h. In control experiments, *L. pneumophila* did not multiply in the macrophage culture medium alone. Thus, all recovered extra- and intracellular bacteria resulted exclusively from intracellular multiplication, since the extracellular bacteria were released after disruption of macrophages loaded with *L. pneumophila*. In each experiment, controls without macrophages were done with the same medium and agent concentrations.

All determinations of CFU were performed on BCYE agar after appropriate dilution in distilled water. Colonies were enumerated after 5 days of incubation at 35°C with 2.5% CO_2 and 95% humidity.

Expression of results. The total number (extra- plus intracellular) of *L. pneumophila*, 24 h after macrophage infection in the presence of serial concentrations of antimicrobial agents, was compared with the total number of *L. pneumophila* at 24 h without agent. Results were expressed by the inhibition ratio: (total *L. pneumophila* at 24 h with agent/total *L. pneumophila* at 24 h without agent) $\times 100\%$.

Thus, values greater than 100% indicated absence of inhibitory effect of the antimicrobial agents and values lower than 100% indicated an inhibitory effect; however, only values lower than or equal to 10% were considered inhibi-

tory (i.e., the number of recovered bacteria with antimicrobial agents 24 h after inoculation was 10-fold less than that without agents). The lowest concentration of agent which had an inhibitory effect on intracellular *L. pneumophila* multiplication was then referred to as the MIEC.

Withdrawal of antimicrobial agents. In some experiments, erythromycin or pefloxacin was withdrawn by repeated washings after 24 h of contact with *L. pneumophila*-infected macrophages. Fresh nutrient medium (TC 199 + 10% normal serum) without antimicrobial agents was added at 24 h. Infected macrophages were then incubated further for various lengths of time, and counts of intracellular and extracellular bacteria were determined at designated intervals.

Combinations of antimicrobial agents. The antimicrobial agents were tested on infected macrophages by the checkerboard method (15) with concentrations lower than the MIECs. The number of points tested for each combination was between 7 and 22.

Statistical analysis. A one-way analysis of variance for unequal group sizes was applied to test for significance, by using a significance level of $P < 0.05$.

RESULTS

MICs of antimicrobial agents against *L. pneumophila*. The MICs of antimicrobial agents for *L. pneumophila* Paris CB 81-13 were found to be within the range of the MICs for the reference strain ATCC 33152 and the eight patient strains (Table 1). The MIC of sulfamethoxazole-trimethoprim was too variable, because of the presence of *p*-aminobenzoic acid in the BCYE agar, which hampers the assessment of sulfamethoxazole (3). The MIC of trimethoprim was 0.4 $\mu\text{g/ml}$ for the strain of *L. pneumophila* we used and within the range of 0.1 to 2 $\mu\text{g/ml}$ for other strains.

Controls. (i) Intracellular multiplication of *L. pneumophila* without antimicrobial agents. The ratio of total bacteria at 24 h without antimicrobial agent to intracellular bacteria at 60 min was equal to 49 ± 36 ($n = 20$). Thus, the spontaneous intracellular multiplication of *L. pneumophila* within macrophages was between 1 and 2 \log_{10} .

(ii) Controls without macrophages. No change of *L. pneumophila* counts was observed in controls without macrophages and without antimicrobial agents over a 24-h period by using the culture medium TC 199 with 10% normal serum (data not shown). With the exception of netilmicin (Table 2), antimicrobial agents added at the concentrations used with macrophages did not induce any change of the bacterial count in the absence of macrophages (Tables 3, 4, and 5).

Effect of antimicrobial agents alone on intracellular multiplication of *L. pneumophila*. (i) **Erythromycin.** The values of the ratio of total bacteria at 24 h with agent to that without agent $\times 100\%$ were less than 10% for concentrations of 1 to 0.05 $\mu\text{g/ml}$, indicating that the number of bacteria at 24 h

TABLE 2. Effect of netilmicin on intracellular multiplication of *L. pneumophila*

Netilmicin concn ($\mu\text{g/ml}$)	Mean % inhibition ratio value \pm SD (n) ^a	
	With macrophages	Without macrophages
5	0.06 \pm 0.003 (2)	0.39 \pm 0.3 (4)
1	3.6 \pm 3 (6)	0.52 \pm 0.45 (5)
0.5	24 \pm 5 (3)	2.7 \pm 0.5 (4)
0.25	138 \pm 57.3 (2)	13.7 \pm 17 (3)

^a Inhibition ratio formula: (total bacteria at 24 h with agent/total bacteria at 24 h without agent) $\times 100\%$. *n*, Number of experiments.

with such concentrations of erythromycin was reduced more than 10-fold (Table 3). With erythromycin concentrations of less than 0.05 µg/ml, there was either low or no reduction of the intracellular multiplication of *L. pneumophila* as demonstrated by ratio values close to 100%.

Thus, the tested concentrations of erythromycin may be classified into two groups: group 1, including concentrations equal to or greater than 0.05 µg/ml, which inhibited the intracellular multiplication, and group 2, with concentrations less than 0.05 µg/ml with no inhibition. No significant differences were observed between values of the ratio within each group ($P > 0.05$), but significant differences between individual values of group 1 and group 2 were demonstrated ($P < 0.001$). Therefore, 0.05 µg/ml was the MIEC of erythromycin for *L. pneumophila*.

(ii) **Rifampin.** An inhibitory effect on the intracellular multiplication of *L. pneumophila* was noted only with rifampin concentrations of 0.01 and 0.005 µg/ml (Table 3). Similar analysis as for erythromycin showed that 0.005 µg/ml was the MIEC for *L. pneumophila*.

(iii) **Doxycycline, sulfamethoxazole-trimethoprim, and pefloxacin.** For doxycycline, sulfamethoxazole-trimethoprim, and pefloxacin, the MIECs for *L. pneumophila* were 0.8, 0.5/0.1, and 0.0625 µg/ml, respectively (Table 4). As for erythromycin and rifampin, concentrations could be classified into two groups. Similar statistical analysis showed no significant differences between values of the ratio within each group ($P > 0.05$), but a significant difference between individual values of group 1 and group 2 ($P < 0.001$ for doxycycline and pefloxacin, $P < 0.01$ for sulfamethoxazole-trimethoprim) was observed.

(iv) **Netilmicin.** An identical analysis could not be performed for netilmicin, since controls without macrophages showed a high reduction of *L. pneumophila* count with netilmicin concentrations equal to or higher than 0.25 µg/ml. With macrophages, the reduction of the intracellular multiplication was observed with concentrations higher than 0.5 µg/ml and to a lesser degree with 0.5 µg/ml (Table 2). Thus, the exact reduction of intracellular multiplication could not be demonstrated, since the reduction observed with macro-

TABLE 4. Effect of doxycycline, sulfamethoxazole-trimethoprim, and pefloxacin on intracellular multiplication of *L. pneumophila*

Antimicrobial agent	Concn (µg/ml)	Mean % inhibition ratio value ± SD (n) ^a	
		With macrophages ^b	Without macrophages ^c
Doxycycline			
Group 1	0.8	0.7 ± 0.2 (3)	80 ± 22 (3)
Group 2	0.4	58 ± 33.8 (3)	137 ± 69 (2)
	0.25	52.7 ± 41.9 (3)	112 ± 41 (3)
	0.05	50.6 ± 21.2 (3)	105 ± 32 (3)
Sulfamethoxazole- trimethoprim			
Group 1	100–20	1.3 ± 0.04 (2)	71 ± 16 (3)
	10–2	1.3 ± 0.03 (2)	89 ± 17 (3)
	0.5–0.1	1.2 ± 0.3 (2)	116 ± 36 (3)
Group 2	0.25–0.05	12.4 ± 4.4 (2)	90 ± 19 (3)
	0.1–0.02	40.1 ± 29.3 (3)	90 (2)
Pefloxacin			
Group 1	10	0.1 ± 0.03 (4)	
	1	1.1 ± 1.9 (8)	73.6 ± 11.8 (5)
	0.5	0.3 ± 0.4 (3)	95.1 ± 15.4 (2)
	0.125	0.3 ± 0.3 (3)	93.1 ± 28.2 (2)
	0.0625	0.6 ± 0.3 (4)	96.1 ± 8.6 (3)
	0.046875	28.7 ± 9.2 (4)	101.2 ± 10.8 (3)
Group 2	0.03125	95.5 ± 17.1 (4)	99.4 ± 18.1 (3)
	0.015625	106.5 ± 54.1 (4)	100 ± 53.7 (3)

^a Ratio formula is given in Table 2, footnote a. n, Number of experiments.

^b For each antimicrobial agent, P is not significant between values within each group (1 and 2); between any individual value of group 1 compared with group 2; $P < 0.001$ for doxycycline and pefloxacin, and $P < 0.01$ for sulfamethoxazole-trimethoprim.

^c For each antimicrobial agent, P is not significant for any individual value.

phages could be caused by the effect of netilmicin on extracellular bacteria being released from disrupted macrophages after intracellular multiplication.

(v) **Cefoxitin.** Cefoxitin had no inhibitory effect on the intracellular multiplication of *L. pneumophila* even at a concentration as high as 64 µg/ml (Table 5), although a slight but nonsignificant reduction of the total number of bacteria at 24 h was observed with a concentration of 64 µg/ml.

Effect of combinations of anti-infectious drugs. Controls without macrophages with the same concentrations of two antimicrobial agents as used with macrophages showed neither reduction nor multiplication of *L. pneumophila* counts at 24 h. The combination of erythromycin and rifampin yielded the lowest fractional inhibitory concentration index (15), 0.6, of the tested combinations (Fig. 1). Pefloxacin combined with erythromycin, rifampin, doxycycline, or netilmicin was also tested. The fractional inhibitory concentration index was 1.5 for pefloxacin and rifampin and

TABLE 3. Effect of erythromycin and rifampin on intracellular multiplication of *L. pneumophila*

Antimicrobial agent	Concn (µg/ml)	Mean % inhibition ratio value ± SD (n) ^a	
		With macrophages ^b	Without macrophages ^c
Erythromycin			
Group 1	1	1 ± 0.4 (4)	87 ± 7.4 (2)
	0.5	0.8 ± 0.4 (4)	87 ± 7.4 (2)
	0.1	3.6 ± 5 (4)	114 ± 22 (4)
	0.05	3.25 ± 2.4 (4)	105.5 ± 42 (4)
Group 2	0.025	36.4 ± 17.1 (7)	96 ± 10 (4)
	0.01	67.5 ± 36.1 (6)	107.6 ± 34 (5)
	0.005	83.25 ± 74 (4)	165 ± 104 (4)
Rifampin			
Group 1	0.01	1.3 ± 1.3 (5)	80 ± 7.8 (3)
	0.005	1.4 ± 1.7 (5)	99.5 ± 48.7 (4)
Group 2	0.0025	88.6 ± 49 (9)	73 ± 26.6 (9)
	0.00125	99.3 ± 52 (7)	71 ± 17 (4)
	0.001	70.1 ± 36 (8)	96.4 ± 26 (5)

^a Ratio formula is given in Table 2, footnote a. n, Number of experiments.

^b For each antimicrobial agent, P is not significant between values within each group (1 and 2); $P < 0.001$ between any individual value of group 1 compared with group 2.

^c For each antimicrobial agent, P is not significant for any individual value.

TABLE 5. Effect of cefoxitin on intracellular multiplication of *L. pneumophila*

Cefoxitin concn (µg/ml)	Mean % inhibition ratio value \pm SD (n) ^a	
	With macrophages	Without macrophages
64	82 \pm 44.8 (5)	89 \pm 1 (3)
8	131 \pm 60.8 (4)	86 \pm 8.4 (2)
1	138 \pm 98.5 (4)	90 \pm 10 (3)
0.1	224 \pm 157 (4)	89 \pm 21 (2)

^a Ratio formula is given in Table 2, footnote a. n, Number of experiments. P is not significant for any individual value.

alone or in combinations for the treatment of Legionnaires disease in humans.

The intracellular activity of netilmicin was difficult to assess because of the effect of this aminoglycoside on extracellular bacteria. However, the intracellular penetration and activity of aminoglycosides are known to be poor. Gentamicin has been shown to be poorly (12) or not (5, 10) effective in an experimental guinea pig infection model and to have no role in the treatment of Legionnaires disease (17).

Thus, antimicrobial agents erythromycin, rifampin and pefloxacin alone are capable of inhibiting the intracellular multiplication of *L. pneumophila*, at concentrations lower than 0.06 µg/ml, and doxycycline and sulfamethoxazole-trimethoprim can cause inhibition at concentrations between 0.1 and 1 µg/ml. Cefoxitin is ineffective. Netilmicin is difficult to assess; its activity remains uncertain. The most interesting point is the good correlation of the activity of antimicrobial agents in a cellular model in vitro and in experimental infection in vivo and also of their respective efficacy in Legionnaires disease.

The comparison of the total number of bacteria at 24 h in the presence of an antimicrobial agent, with the number of intracellular bacteria at 60 min regarded as the intracellular inoculum, may supply information on intracellular killing effect of the antimicrobial agents. For the three most active agents, erythromycin, rifampin, and pefloxacin, 5 to 20% of the intracellular bacterial inoculum remained viable at 24 h at concentrations equal to or up to 20-fold higher than the MIECs (data not shown). This reduction of the intracellular inoculum may be caused by actual intracellular bactericidal activity of the agents or by combination of bactericidal mechanisms of phagocytes with the effect of agents. Thus, the comparison with the bactericidal activity of antimicrobial agent in a usual acellular system remains questionable. However, this reduction was slightly greater with pefloxacin than with the other agents; this could explain, at least in part, the delayed multiplication of *L. pneumophila* after withdrawal of pefloxacin.

In the intracellular model, combinations of erythromycin and rifampin, and of pefloxacin and other agents, yielded an additive effect. This is likely to be of clinical significance in the treatment of Legionnaires disease. The use of rifampin alone, which is a very potent antibiotic treatment in experimental infection (5), is hampered by the theoretical risk of emergence of resistant mutants (3). These facts sustain the use of combinations of erythromycin and rifampin in critically ill patients. Other combinations, such as new quinolones and rifampin, may also be assessed in such cases.

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